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A New *Lea* Gene is Induced During Osmopriming of *Capsicum annuum* L. Seeds

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Abstract: The aim of this study was to characterize the transcriptional expression patterns of a new *lea* gene isolated in a previous work from *C. annuum* cv. caballero seeds when osmoprimed with PEG and GA_3. *Capsicum annuum* is one of the main horticultural crops in México and routinely their seeds have problems when germinating. To correct this problem, osmopriming treatments based on PEG and GA has been used to improve their vigor. Osmopriming is a strategy developed to improve vigor during seed storage, which causes a reduction in germinability and seedling establishment. Osmopriming consists of the pre-imbibition of seeds in a solution containing an inert osmotic agent such as polyethylene glycol (PEG). In combination with PEG, several other compounds such as gibberellic acid (GA) can be used in order to improve the vigor of seeds. Several ESTs with high induced expression in the osmopriming treatment displayed high homology to LEA proteins and one of them corresponded to a complete cDNA coding a new LEA protein of 73 amino acids (*Calea* 73 gene). This gene was highly induced in osmoprimed treatments in which KNO_3 instead of GA_3 was used in combination with PEG on *C. annuum* cv. caballero seeds. To our knowledge this is the shortest *lea* gene reported so far.

Key words: LEA proteins, *Capsicum annuum*, osmopriming, abscisic acid, water stress

INTRODUCTION

Pepper (*Capsicum annuum* L.) cv. caballero seeds commonly show problems to germinate vigorously and thus, several osmopriming treatments have been proposed to correct this agronomic deficiency. The use of PEG 6000 alone or in combination with either GA_3 or KNO_3, has shown to improve the vigor of germinating seeds (Cortez-Baheza *et al*., 2007). The quality of dry seeds is important in agriculture, since seeds are often the starting material for crop production and crucial for achieving a good harvest. Several aspects of seed quality influence agricultural performance, such as total emergence, the rate and uniformity of emergence, emergence under suboptimal conditions and seed longevity. To improve these aspects several priming treatments have been developed. During priming treatments seeds are allowed to take up water and partially start their germination-related processes, but emergence of the radicle is prevented to avoid the loss of desiccation tolerance needed for subsequent drying, storage and marketing of the treated seeds. Priming treatments are also used to synchronize the germination of individual seeds (Heydekker *et al*., 1973). Because priming promotes the initiation of several germination-related processes, it induces causes faster germination and field emergence, especially under adverse field conditions (McDonald, 2000). To prevent radicle protrusion, water uptake may

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either be limited by imbibition in an osmotic solution (osmopriming) instead of water or by restricting the period of germination on water and drying the seeds prior the radicle protrusion (Soeda *et al*., 2005). During osmopriming only a subset of events occurs, in comparison with germination on water, as previously demonstrated at protein level (Gallardo *et al*., 2001).

The expression of certain genes during maturation and seed processing, as well as osmopriming, results in an altered physiological state and affects seed quality. Therefore, the genes whose expression levels are different among osmopriming treatments might be useful as markers for identifying important processes in the improvement of seed vigor. It has been shown that several late embryogenesis abundant (*lea*) genes are strongly induced in *C. annuum* cv. caballero seeds when osmoprimed with PEG and GA_3 (Cortez-Baheza *et al*., 2007). LEA proteins cover a number of loosely related groups of proteins whose precise function is unknown (Wise, 2003). LEA proteins were first characterized in cotton plant (Dure *et al*., 1981) and are produced in abundance during seed development, comprising up to 4% of cellular proteins (Goyal *et al*., 2005). Their expression is linked to the acquisition of desiccation tolerance in orthodox seeds, pollen and anhydrobiotic plants, but many LEA proteins are induced by exogenous factors such as cold, osmotic stress or ABA and in some cases they are expressed constitutively (Chung *et al*., 2003; Goyal *et al*., 2005). LEA proteins have received a special attention due to its apparently important role in desiccation tolerance in several life forms (Berjak, 2006; Tunnacliffe and Wise, 2007). In this study, we report the structural characterization of a complete cDNA corresponding to a new *lea* gene (*Calea* 73) from *C. annuum* cv. caballero seeds osmoprimed with PEG and GA_3. Expression studies of this gene in seeds and vegetative tissues during four months with different osmotic stresses showed that *Calea* 73 was induced in seeds osmoprimed with PEG and KNO_3 and in plants incubated under cold stress and treated with exogenous ABA and slightly induced in some organs during specific drought conditions. Several aspects related to the possible biotechnological applications of this new gene are discussed.

MATERIALS AND METHODS

Source of seeds: The seeds of *Capsicum annuum* cv. caballero were purchased from SAKATA seeds de México (Tesistán, Jalisco, México). This study was conducted from September 2006 to June 2007.

Chemicals: Polyethylene glycol (PEG) 6000, Potassium nitrate and gibberellic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Osmopriming of seeds: The seeds of *C. annuum* cv. caballero were first dried at 25°C in an incubator (FELISA, Guadalajara, Jalisco, México), then were homogenized in a pneumatic sorter (Manufacturing Company Hoffman, Albany, Or, USA) and finally maintained in flasks at 12% of relative humidity in a growth chamber (Cenviron, Winnipeg, Manitoba, Canada) until their use in osmopriming experiments. Osmopriming treatments were carried out as following: 10 g of seeds were placed in a Petri dish and then the seeds were imbibed in 10 mL of PEG 6000 (500 ppm) + gibberellic acid (500 ppm), or PEG 6000 (500 ppm) both equivalent to -0.01 MPa as measured by thermocouple psychrometry. The time of imbibition was 9 h and then the seeds were washed with distilled water at 25°C. Both treatments were further used in construction of a subtraction suppression hybridization library (CLONTECH PCR-Select™ cDNA subtraction kit; Clontech, Palo Alto, CA, USA) enriched in up-regulated genes for GA_3.

Plant material and growth conditions: Pepper seeds were surface-sterilized in 10% (v/v) sodium hypochlorite for 10 min, rinsed in running tap water for 2 h, sown on water-saturated paper towels and germinated in the dark at 27°C and 100% relative humidity. After 5 days, seedlings were selected for uniform size and transplanted to pots with sterilized soil. Plants obtained by this method were used in ABA and cold stress experiments as following: ABA treatment was carried out by adding 0.1 mM ABA to the irrigating water and by spraying an ABA solution (0.1 mM) to the aerial regions. When collected, tissues were frozen immediately in liquid nitrogen and stored at -80°C until used for RNA extraction. Cold stress experiments were carried out by incubating four-leaf stage plants at 4°C for 3 days and then tissues were collected and processed as in ABA treatments for RNA extraction.

Drought tolerance experiments: A soil mixture was prepared (50% fine sand: 50% sandy clay loam) and its field capacity determined according to Daubenmire (1974). Sixteen plastic bags (22.2 cm diameter and 35 cm high) were filled with this soil mixture, placing one soil psychrometer (model PCT-55; Wescor, Inc., Logan, Utah) at the half (17.5 cm high) of every bag. The pots were then watered to field capacity, fertilized once with 200 mL of Hoagland´s solution (Hoagland and Arnon, 1950) and three seeds were buried in the center of these pots (2 cm depth) and permitted to establish for a month. After the

establishment period, the containers were cleared to one plant per pot selecting even sized plants with experimental homogenization purposes. At this time, the watering was suspended to eight plants (water stress treatment) and the soil water potential monitored every three days in both stressed and non-stressed for the rest of the experiment connecting the thermocouple psychrometers to a microvoltmeter (model HR-33T; Wescor, Inc., Logan, Utah). Leaf water potential of the water-stressed and control plants were measured at 6:00 h am and 13:00 h pm punching and placing round leaf discs in C-52 sample chambers (Wescor, Inc., Logan, Utah) connected to the Wescor HR-33T microvoltmeter. Complete plants were detached extracted from the bags when they reached water potentials of -0.22, -0.47 and -1.5 MPa (value close to the permanent wilting point). A group of plants attaining a water potential of -1.5 MPa were then re-watered to field capacity and permitted to stabilize before they were detached from the bags. All removed plants were stored at -80°C until RNA extraction.

Isolation of total RNA: RNeasy plant mini kit (Qiagen, Hilden, Germany) was used to extract the total RNA from 3 g of osmoprimed seeds. RNA purified by RNeasy column was analyzed for integrity and size by formaldehyde agarose gel electrophoresis, while concentration and purity of RNA were determined by $OD_{260/280}$ value.

Slot blot analysis: The Northern blot analysis was essentially carried out as mentioned in Sambrook *et al.* (1989). For each treatment, 15 μg of total RNA were used.

DNA sequencing and database comparison: The nucleotide sequence of *Calea*73 gene was determined using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT, USA). The sequence was deposited in Genbank (accession number DQ902577.1). On-line database comparisons were performed using Blastx algorithm (Altscchul *et al*., 1990) from National Center for Biotechnology Information (NCBI). The LEA protein sequences used in the analysis for comparison purposes with *Calea* 73 have the following accession numbers: *A. thaliana* (NM_112632), *G. hirsutum* (P09443), *G. max* (AF117884.1), Dehydrin *C. annuum* (AY225438.1) and *H. annus* (X59700).

RESULTS

Isolation and characterization of *Calea* 73 cDNA sequence: A subtracted cDNA library enriched with *C. annuum* cv. caballero genes up-regulated after osmopriming treatment with GA_3 in addition to PEG was generated by suppression subtractive hybridization (Diatchenko *et al*., 1996). One clone of the library showed high similarity to *lea* gene sequences (Cortez-Baheza *et al*., 2007). The clone contained an insert of 368 bp, including a poly (A) end which indicated the 3′end of the cDNA. Additionally, this clone showed a complete Open Reading Frame (ORF) encoding a putative LEA protein of 73 aminoacids and 23 bp at 5′end, which was further confirmed by 5′-RACE analysis. Thus, this SSH-library clone corresponds to a full-size cDNA with an ORF of 219 bp and was named as *Calea* 73 (Fig. 1A). Phylogenetic analysis utilizing other LEA protein

A

5′ACGCGGGGGAGAGAATAAAGGAG**ATG**GGAGAGAAGGCGGAGG
CGGAGACGGAGGAACATGTAAACTGGGCAAAAGAGAAGGCGAAA
GAAGGATACGAAAGCGCAAAGAACAAAGCAGGAGAGACATTGGAG
GAAGCTAAAGAAAGTGTAGCTTCAAATTTGGAGTCAGCTAAGGAAA
CTGCTAAAGAGAAAACTAAGGAGATCAAGGAGAATATAGCTGGGAA
AAAAAGAGATGAGGAGCTG**TAG**AAAGTTTTTCTTTTTGTTATTGGA
GTTTACATGTATGTTTGTATTTTGCATTATCATAGTTTATATGTTGT**AA**
TAGAAAGCTGATGTTTTTGTTTTTCCAAAAAAAAAAAAAAAAAAAA
AA 3′

B

MGEKAEAETEEhvnwakekake**GYESAKNKAGETLEEAKESV**
ASNLESaketakektke**IKENIAGKKRDEEL**

Fig. 1: Nucleotides and amino acids sequence of *Calea* 73 gene. Panel A, nucleotide sequence of the complete cDNA isolated from *C. annuum* cv. Caballero plants, indicating in bold and underlined the first and stop codon of the open reading frame and only in bold a putative polyadenilation signal. Panel B, amino acids sequence of the putative LEA protein. Underlined are indicated two putative 11 mer motifs typical in LEA proteins of group 3

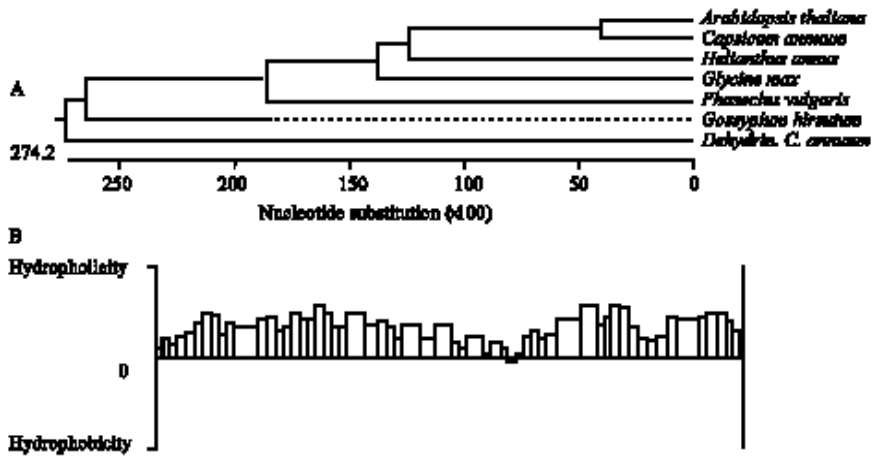

Fig. 2: Phylogenetic tree and aminoacids profile of the putative LEA protein encoded by *Calea* 73 gene. Panel A, phylogenetic tree obtained comparing several LEA proteins reported elsewhere. Panel B, amino acids profiles of the putative LEA protein, using PROTEAN tool of DNASTAR

sequences showed that the putative LEA protein encoded by *Calea* 73 showed a major similarity with a LEA protein of *Arabidopsis thaliana* (Fig. 2A). Additionally, this protein displayed two putative 11-mer motif previously identified in group 3 LEA proteins (Fig. 1B) and showed a high hidrophilicity profile (Fig. 2B) which is a typical feature in the majority of LEA proteins (Goyal *et al*., 2005).

Analysis of expression of *Calea* 73 gene in seeds and vegetative tissues: Characterization of the *Calea* 73 expression in seeds of *C. annuum* cv. caballero under osmopriming treatments indicated that in addition to the induced expression at the transcriptional level under PEG+ AG_3, the treatment with PEG+ KNO_3 also induced a similar expression of this gene when these seeds were osmoprimed (Fig. 3).

A feature of *lea* genes is their high transcriptional expression in late embryogenesis stages during seeds development, but once germinating; their expression is highly diminished or even completely disappears (Campos-Alvarez *et al*., 2002; Grelet *et al*., 2005). This behavior was displayed by *Calea* 73 in this study because its expression was not detected in plants 7 days post-germination (Fig. 4B, lanes 1-4). Expression of *lea* genes often appears to be abscisic acid-dependent (Xiao *et al*., 2007). Thus, to evaluate whether *Calea* 73 is induced by abscisic acid (ABA) in *C. annuum* cv. caballero plants were grown for 4 months and several experiments were carried out to address this question. It was shown that *Calea* 73 gene was induced at 1 minute, 16, 24 and 48 h after ectopic application of ABA on leaves of 15 but not on 7 days-old plants of *C. annuum* cv. caballero (Fig. 4A and B, lanes 5-8). Interestingly, plants

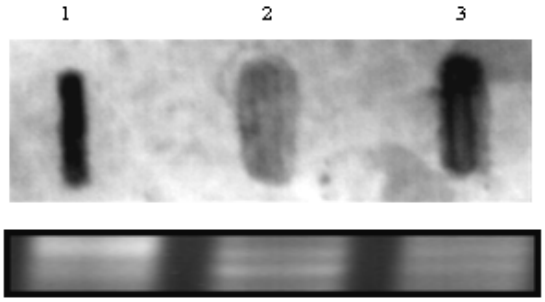

Fig. 3: Expression of *Calea* 73 gene on *C. annuum* cv. Caballero seeds treated with several osmopriming solutions. Lane 1, PEG 6000+GA3; Lane 2, only PEG 6000; Lane 3, PEG 6000+KNO_3. The sequence of the *Calea* 73 open reading frame was used as a probe

on which no ABA was applied did not display any detectable *Calea* 73 expression during these same periods (Fig. 4A and B, lanes 1-4). In addition, it is worth mentioning that the expression of *Calea* 73 was detected in low levels in 2 month-old pepper plants, but no during other growing stages on several organs as leaves, root, stem, flowers and fruits regardless of ABA applications (not shown).

Another reported feature of several *lea* genes is their expression under cold stress in plants (Thomashow, 1998; Cumming, 1999; Xiao *et al*., 2007). In this study, cold stress in 2 months-old *C. annuum* cv. caballero plants displayed an induction of *Calea* 73 in root, but not in leaves or stem after 16 h of incubation at 4°C (Fig. 5C). Expression of *Calea* 73 was also slightly detected after

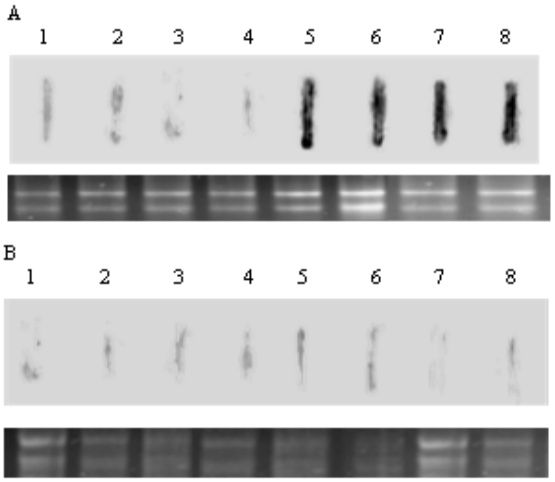

Fig. 4: Expression of *Calea* 73 gene on 7 and 15 days-old plants of *C. annuum* cv. Caballero. Panel A, 15 days-old plants. Panel B, 7 days-old plants. On both panels, lanes 1-4 no ABA application (control) and lanes 5-8, RNA extracted from pepper plants in 1 min and 16, 24 and 48 h after ABA application. In control plants, the same times were used. Visualization of rRNA was used as a quantification control. The sequence of the *Calea* 73 open reading frame was used as a probe

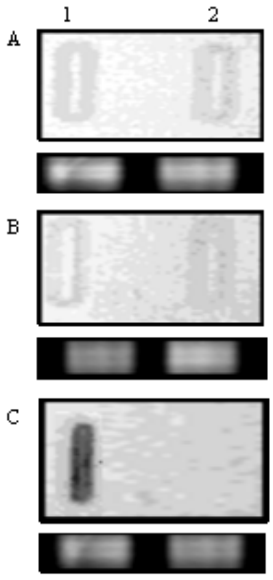

Fig. 5: Expression of *Calea* 73 gene in different organs of *C. annuum* cv. Caballero plants during cold stress. Panels A, B and C, RNA extract from leaves, stems and roots, respectively. Visualization of rRNA was used as a quantification control. The sequence of the *Calea* 73 open reading frame was used as a probe

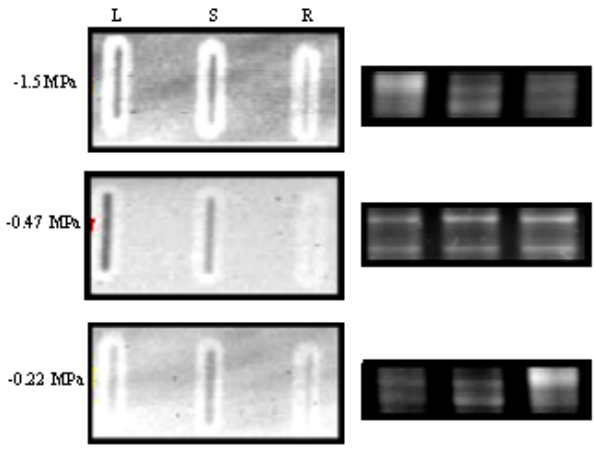

Fig. 6: Expression of *Calea* 73 gene in different organs of *C. annuum* cv. Caballero plants during water deficit stress. L, S and R, corresponds to RNA extracted from leaves, stems and roots, respectively. Visualization of rRNA was used as a quantification control. The sequence of the *Calea* 73 open reading frame was used as a probe. Water stress experiments were carried out as mentioned in materials and methods

the same incubation time on 3 months-old plants on leaves, stem and root (not shown). No expression of *Calea* 73 was detected in other growth stages of the pepper plants evaluated.

Expression of *Calea* 73 on water stress conditions: As mentioned before, several reports indicated that LEA proteins are implicated in water deficit stress response (Xu *et al*., 1996; Maqbool *et al*., 2002; Goyal *et al*., 2005). Thus, the expression of *Calea* 73 was evaluated in *C. annuum* cv. caballero plants on 2 months-old plants. As shown in Fig. 6, *Calea* 73 was slightly more induced on leaves than on stem and roots, when water deficit was increased from the control plants (-0.22 MPa) to plants near to the permanent wilting point (-1.5 MPa). Expression of *Calea* 73 on stems was low and apparently not induced when increasing the water stress level, while the lowest expression was detected in roots (Fig. 6). No significant change in *Calea* 73 expression was detected in rehydrated plants from -1.5 to -0.22 MPa (data not shown).

DISCUSSION

Osmopriming treatments using PEG in combination with GA_3 or KNO_3, have shown to be good approaches to re-vigorate pepper seeds for commercial purposes (Cortez-Baheza *et al*., 2007). Seed maturation is characterized by

a desiccation process, in which, several proteins referred to as LEA accumulate in the embryo (Baker *et al*., 1988; Hughes and Galau, 1989). As mentioned before *lea* genes are high expressed in developing embryos during seed maturation (Baker *et al*., 1988; Galau *et al*., 1986) and environmental stresses (such as cold, salt and water stress) and by application of ABA to the vegetative tissues (Chung *et al*., 2003; Choi *et al*., 1999; Cohen and Bray, 1992; Godoy *et al*., 1990; Aguado-Santacruz, 2006). In this study a structural characterization has been done for a previously isolated complete cDNA encoding a new *lea* gene (*Calea* 73) induced in *C. annuum* cv. caballero seeds during osmopriming with PEG + GA_3 (Cortez-Baheza *et al*., 2007). The *Calea* 73 gene displayed a typical hydrophilic profile and some features reported in group 3 LEA proteins as two putative 11-mer motifs TA (EQ) AAK (EQ) KAXE (Goyal *et al*., 2005; Tobe *et al*., 2004). Representatives of this group of genes also occur in prokaryotes (Dure, 2001), protozoans, nematodes and rotifers (Tunnacliffe and Lapinsky, 2003).

Based on the data presented here, we propose that *Calea* 73 is a new *lea* gene of the group 3 of LEA proteins; to our knowledge it is the shortest one reported so far. On the other hand, our results showed that *Calea* 73 was induced on both pepper seeds osmopriming treatments and in some organs in vegetative tissues during some growing stages in response to cold and drought stresses and ABA treatment. Besides of being induced by PEG + GA_3, the *Calea* 73 gene was also stimulated by PEG+KNO_3, which indicates that this gene is expressed during osmopriming regardless the osmotic solution used. Saline soils contain multiple types of soluble salt components, exerting different effects on the initial growth of plants (Younis and Hatata, 1971; Redmann, 1974; Hardegree and Emmerich, 1990; Tobe *et al*., 2002, 2003). These salts have effects on cell membranes and cell walls that affect the water potential of the cytosol and cellular extensibility and thus, may affect seed germination and seedling growth (Tobe *et al*., 2004). In this study, it was evident that the response of *Calea* 73 gene to the abiotic stresses evaluated and ABA applications in *C. annuum* cv. caballero plants was stage-growing and organ dependent, especially after 15 days and 2 months post-germination. Although at low levels, even in 2 months-old plants the *Calea* 73 expression could be detected without the ectopic application of ABA. These results suggest a highly regulated transcriptional control of *Calea* 73, in which at least: temporal, spatial and hormonal components are involved as in other systems reported elsewhere (Bray, 1997; Vicient *et al*., 2000). The exact nature of the factors determining this regulation and the functional implications of the different patterns of expression of *Calea* 73 remain to be elucidated. The fact that the rehydration process of pepper plants in our study did not result in a significant change in *Calea* 73 gene expression, suggests that the mRNA corresponding to this gene is highly stable under the evaluated conditions, although the importance of this control is unclear so far.

CONCLUSION

Taking together our results showed that *Calea* 73 is a *lea* gene corresponding to group 3, which is induced in vegetative tissues in pepper plants in response to cold, drought and ABA applications, in a growing-stage depending manner. It should be interesting to study the overexpression of this gene in order to evaluate its possible biotechnological application as a new gene conferring drought tolerance in agriculture.

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